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Distribution of [^{14}C]diazepam associated with two different types of small unilamellar liposomes in mice

U. Müller and K. Munz

Institute of Pharmacology, University of Zürich, Zürich (Switzerland)

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Summary

Distribution and localization (on macroscopic and electron microscopic level) of two [^{14}C]diazepam-liposome carrier systems, consisting of small unilamellar vesicles with either phosphatidic acid or sphingomyelin as characteristic lipid constituents have been studied following i.v. injection into mice. Liver and adipose tissue were the main target tissues. In contrast to distribution of free drug no diazepam was detected in the brain. Furthermore, no redistribution to the brain or peripheral organs has been observed during 24 h. Comparative ultrastructural investigations of the liver revealed that the labeled diazepam was incorporated mainly into parenchymal cells with no preference for either of the two carrier systems. The elimination pattern of diazepam-labeled liposomes and of the lipid-labeled liposome systems were almost the same. Incorporation of radioactivity into the membrane of hepatocytes could be excluded. An interesting site of localization of radioactivity in these cells was the nucleus and its membrane. To a lesser extent [^{14}C]diazepam was also localized in Kupffer cells.

Introduction

Liposomes are under investigation as carriers for local administration of drugs, as devices to achieve sustained release of encapsulated drugs or to solubilize lipophilic drugs for i.v. injection. After i.v. injection liposomes either degrade rapidly in the circulation (Kirkby et al., 1980; Senior and Gregoriadis, 1982; Scherphof et al., 1983a) or they are taken up mainly by the cells of the liver. Of the various cell types populating the liver, Kupffer cells (macrophages) and parenchymal cells (hepatocytes) are involved in liposome uptake. Liposome distribution into these distinct cell types is dependent on their size, charge and lipid composition (Gregoriadis and Neerunjun, 1974; Roerdink et al., 1983; Spanjer et al., 1983; Dijkstra et al., 1984). Large liposomes are taken up preferentially through endocytosis by Kupffer cells, as they cannot reach the parenchymal cells through the 100 nm fenestration in the sinusoidal endothelium (Wisse, 1970; Scherphof et al., 1983b). Small liposomes associate to a large extent with parenchymal cells (Hoekstra et al., 1978; Poste et al., 1982; Roerdink et al., 1984). The liver is also the major organ responsible for processing and elimination of systemically administered liposomes which has been investigated biochemically

Correspondence: U. Müller, Institute of Pharmacology, University of Zürich, Gloriastrasse 32, CH-8006 Zürich, Switzerland.

and morphologically by Roerdink et al. (1984), Dijkstra et al. (1985), Scherphof et al. (1985) and Waser et al. (1987). Nevertheless, important questions (e.g., are hepatocyte-associated, small unilamellar liposomes truly internalized or are they affixed to the cell surface or do they fuse with the cell membrane) remain to be further cleared.

We studied distribution and localization of the lipophilic drug diazepam and cholesteryl hexadecyl ether associated either with negatively charged or sphingomyelin containing small unilamellar lipsomes in the liver after intravenous administration in mice by electron microscopic autoradiography.

In our work [^{14}C]diazepam was used twofold as a marker. First, the centrally acting drug was used as clue for viability of the liposome carrier systems as slow release systems. Second [^{14}C]diazepam as a lipophilic substance was also utilized to follow the fate of the carrier material in the target tissue in comparison to the tightly bound lipid marker [^{3}H]cholesteryl hexadecyl ether.

Materials and Methods

Materials

Phosphatidylcholine and phosphatidic acid were purchased from Lipid Products, Nutfield Nurseries, South Nutfield, U.K. Sphingomyelin, cholesterol, tannic acid and Durcupan ACM (Araldite) were purchased from Fluka AG, Buchs, Switzerland. [^{14}C]diazepam (specific activity of 186 μCi/mg) was a gift from Hoffmann-LaRoche & Co, Basel, Switzerland, delivered as solution for i.v. administration in human therapy. [^{3}H]cholesteryl hexadecyl ether was purchased from NEN Research Products, Du Pont de Nemours International S.A. Nuclear Research Emulsion L4 was obtained from Ilford Ltd., Ilford, U.K.

Preparation of [^{14}C]diazepam phospholipid vesicles (liposomes) and [^{3}H]cholesteryl hexadecyl ether liposomes

Two batches of different lipid composition were used, containing either phosphatidyl choline and phosphatidic acid (8 : 2 mol), system I, or phosphatidyl choline, sphingomyelin and cholesterol (4 : 4 : 2 mol), system II. The liposomes were prepared according to the method described by Müller et al. (1983). In brief, the lipids were dissolved in chloroform/methanol (1 : 1, v/v) in a round flask. The solvents were then removed under reduced pressure on a rotary evaporation apparatus leaving a thin film on the glass wall. Thereafter, the labeled diazepam (76 μCi/batch) in PIPES buffer (pH 7.2) containing 30 mM of sodium cholate was added. Brief mechanical shaking allowed the lipid–drug–detergent mixture to form a dispersion which was left for equilibration. This dispersion was centrifuged through a Sephadex G50 column adapted into a GSA rotor of a Sorvall centrifuge. The Sephadex was equilibrated too with the above buffer and the void volume separated by centrifugation. The final volume varying from 1.0 to 1.3 ml contained 48–50% of the initial activities. Lipid-labeled liposomes were prepared as above, but [^{3}H]cholesteryl hexadecyl ether was added instead of [^{14}C]diazepam as marker. The particle size of the liposomes as determined by transmission electron microscopy after negative staining was between 50 and 70 nm.

Animal experiments and preparation of cryosections

Male albino mice strain ICR were used with an average weight of 20 g. Groups of 8 animals were injected each with 100 μl of [^{14}C]diazepam liposomes (3–4 μCi) into tail veins. After appropriate time intervals, namely 5 min, 6 h and 24 hours, 6 animals (3 for each liposome system) were killed and submerged in a mixture of hexane/dry ice (−78 °C). The remaining two animals of each group were used for electron microscopic experiments. In addition, 6 animals were injected with the lipid labeled liposomes and killed at appropriate time intervals.

The frozen animals were then embedded in 5% sodium carboxy-methyl cellulose in a metal frame at −78 °C. Whole body sagittal sections were adhered to Scotch tape 3M 810 and freeze dried at −20 °C for 48 h.

Whole body autoradiography and quantitative determination of radioactivity in the organs

Sections were exposed to Kodak X-ray film DEF-5 at −20 °C up to 12 weeks and the films

were developed in Kodak D-19. Blackening of the film was measured using a transmission densitometer (Macbeth TD-504) and compared with standards, containing a scale of dilution concentrations with [^{14}C]glucose, as described by Cross et al. (1974).

Electron microscopy: preparation of liver tissue and autoradiographs

Freshly excised liver tissue was cut into small pieces of about 1 mm^3 and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h at 0 °C. After 3 washes in cacodylate buffer tissue pieces were resuspended in 1.5% tannic acid in cacodylate buffer, pH 7.2 for 1 h at 0 °C. Before immersion in 1% osmium tetroxide for a further hour, samples were washed extensively in cacodylate buffer to eliminate any free tannic acid in the solution. After removal of osmium tetroxide and two further washes in buffer, the liver pieces were dehydrated gradually in ethanol and embedded conventionally in Durcupan ACM (Araldite).

Ultrathin sections were placed on 200-mesh copper grids which had been coated with Collodium film and reinforced with carbon. Then 6–12 grids were mounted onto glass slides with a touch of glue on two sides. These mounted grids were coated with Ilford L4 emulsion by the dipping method (Rogers, 1979). Coated slides were thouroughly air dried and stored in the dark for 20 days in plastic boxes at −20 °C. Development of the autoradiographs was carried out according to standard methods. After prolonged washing in distilled water, the wet grids were immediately stained with uranyl acetate and lead citrate. Autoradiographs were observed in a Philips 400 T electron microscope.

Results

Whole body autoradiography

A typical distribution pattern of [^{14}C]labeled diazepam associated with small unilamellar liposomes in the organs of mice is shown in Fig. 1. The autoradiograph shows that [^{14}C]diazepam, 5 min after i.v. injection into mice, reaches the brain only in a negligible quantity. In contrast, the appearance of radioactivity (RA) in the liver is conspicuous. Furthermore, the adipose tissue of the neck, the salivary gland and the mucosa of the pharynx and the nose represent remarkable sites of incorporation of RA. In the excretion organs

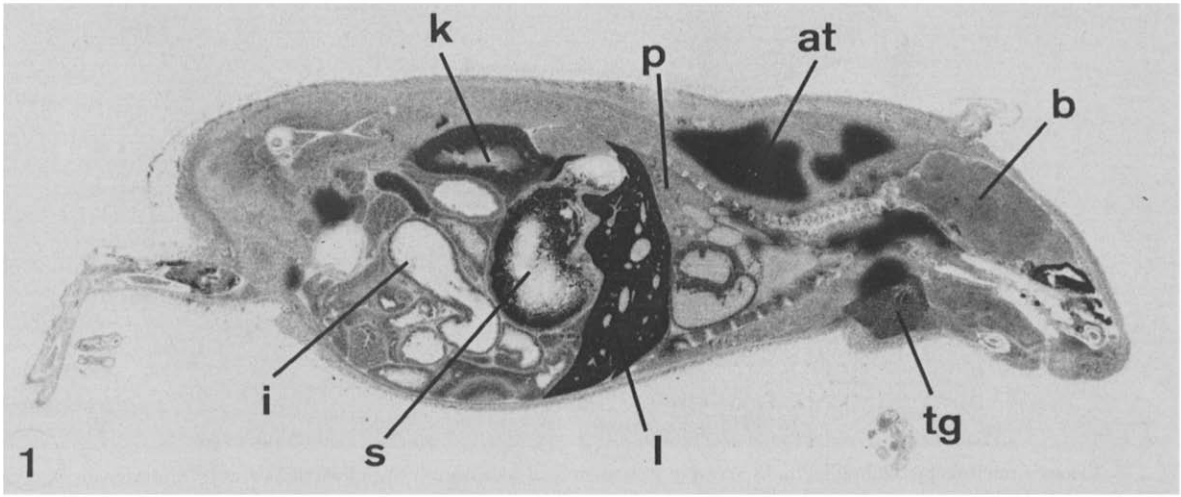


Fig. 1. Whole body autoradiograph of a mouse 5 min after i.v. injection of phosphatidic acid containing liposomes (system I). Most of the radioactivity (silver grains) is found in liver (l), adipose tissue (at), stomach (s) and kidney (k). Only little radioactivity is detected in lungs (p) and in brain (b); i, intestine; tg, thyroid gland.

kidney, gall bladder and urinary bladder RA is already visible. No RA is found in the lungs. Within the first 30 minutes, i.v. administration of liposomes leads to accumulation of RA in liver, kidney and intestine. In the adipose tissue of the neck, RA diminishes significantly. No redistribution of released free diazepam within the observed time intervals can be detected in the body as the brain contains still minor amounts of [^{14}C]diazepam.

Electron microscopy

Figs. 2–8 show typical situations of mouse liver invaded by one of the two [^{14}C]diazepam liposome systems, either the negatively charged phosphatidic acid containing carrier system I or the sphingomyelin containing system II, being neutrally charged. A semiquantitative analysis is done on the basis of an average number of 125 autoradiographs showing silver grains for both liposome systems administered and for each time interval (namely 5 min, 6 and 24 h).

At all time points RA of system I and system II is found incorporated mostly into the parenchymal cells with no preference of either of the two carrier systems (Fig. 2). Precise attribution of silver grains to intracellular structures is difficult, but incorporation into the membrane of the parenchymal cells can be excluded. For both carrier systems and at all 3 time points, a remarkable number of silver grains could be detected in the nuclei of the parenchymal cells (Fig. 3) and their membranes. Other interesting sites of localization of the silver grains are the glycogen-storing regions in the cytoplasm of these cells (arrow in Figs. 3 and 4). Furthermore, cluster formation of RA is detected

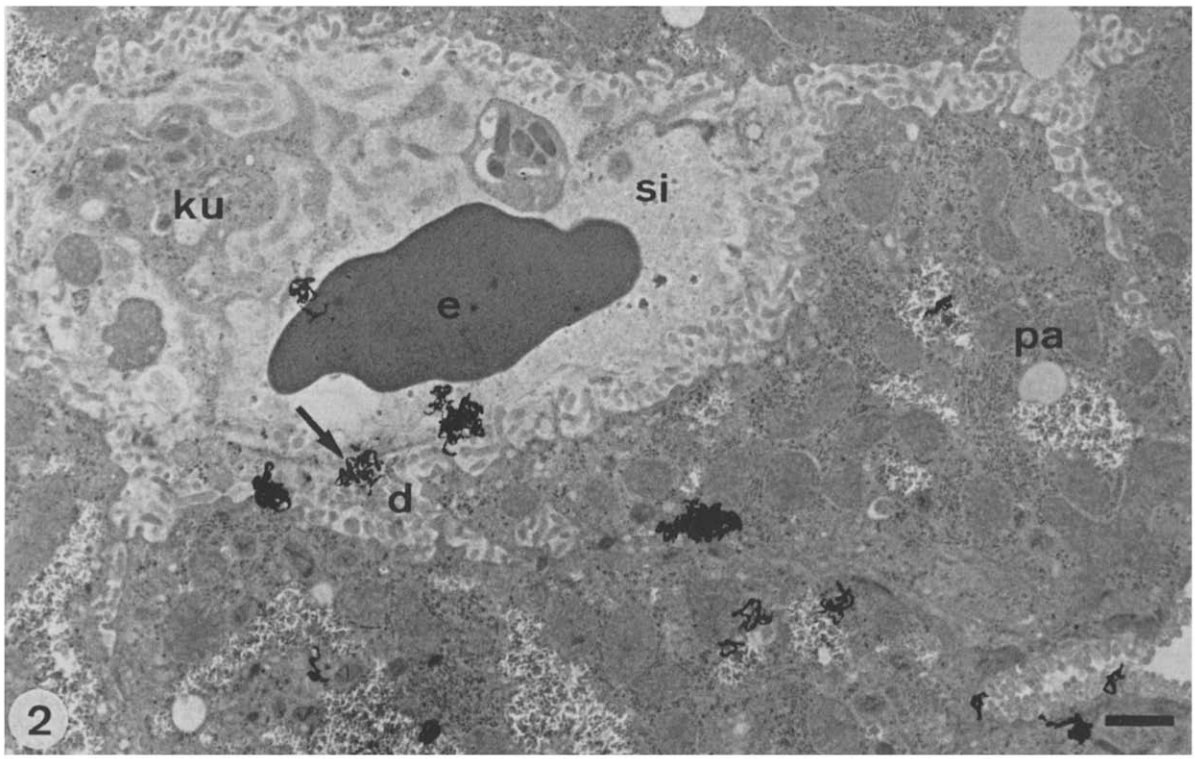


Fig. 2–8. Electron microscopic autoradiographs showing typical sites of localization of radioactivity from ^{14}C-diazepam associated with either phosphatidic acid- or sphingomyelin-containing small unilamellar liposomes (systems I and II) after i.v. injection in mice.

Fig. 2. System I: most of the labeled diazepam is found incorporated in parenchymal cells (pa) and in the space of Disse (d). Fewer silver grains are also detected in the sinusoidal compartment (si); e, erythrocyte; ku, Kupffer cell. Bar = 1 μm.

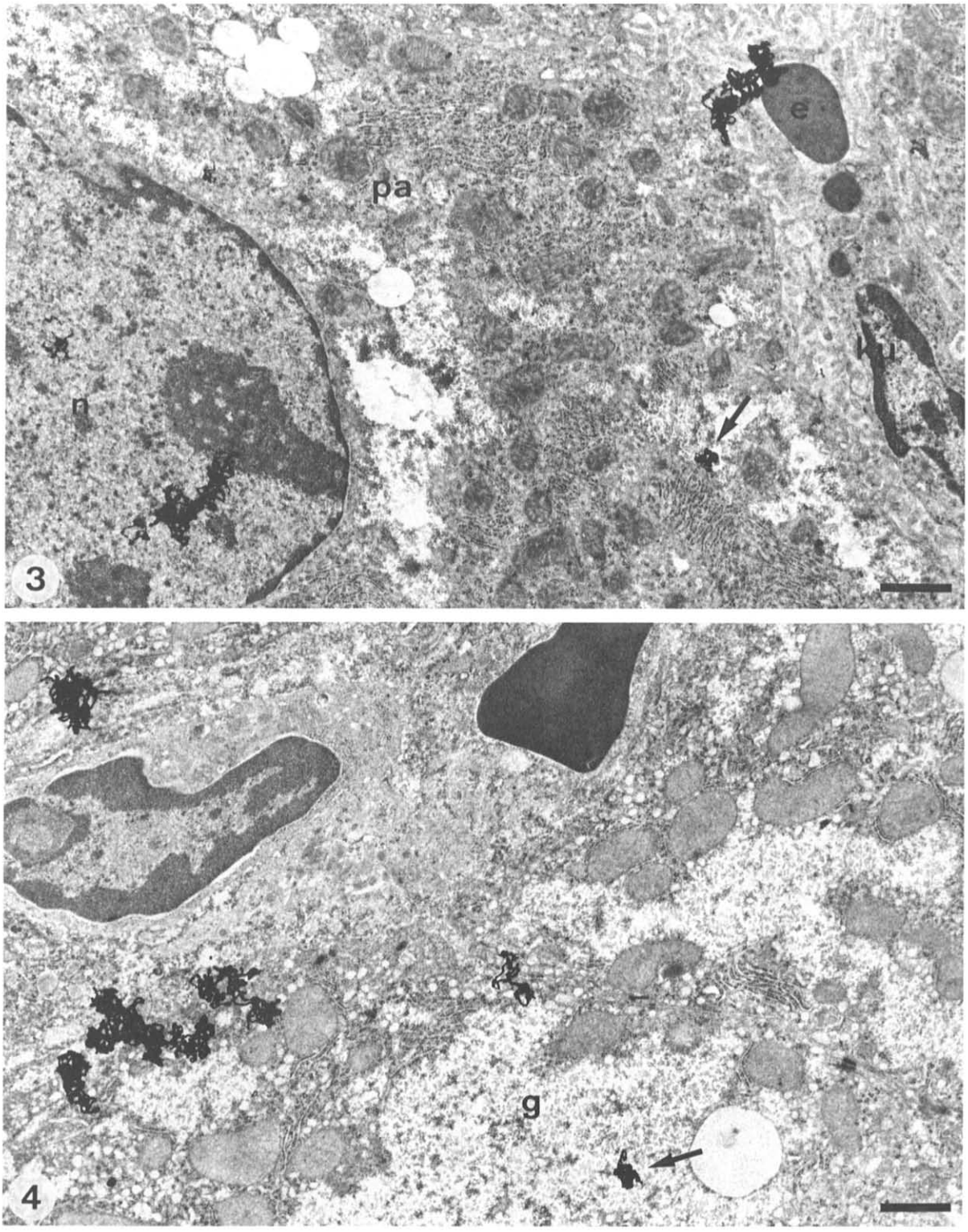
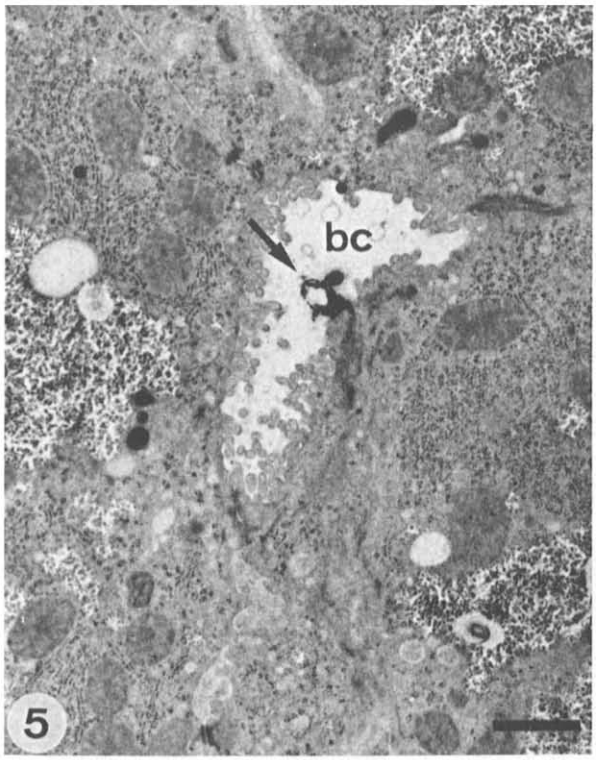
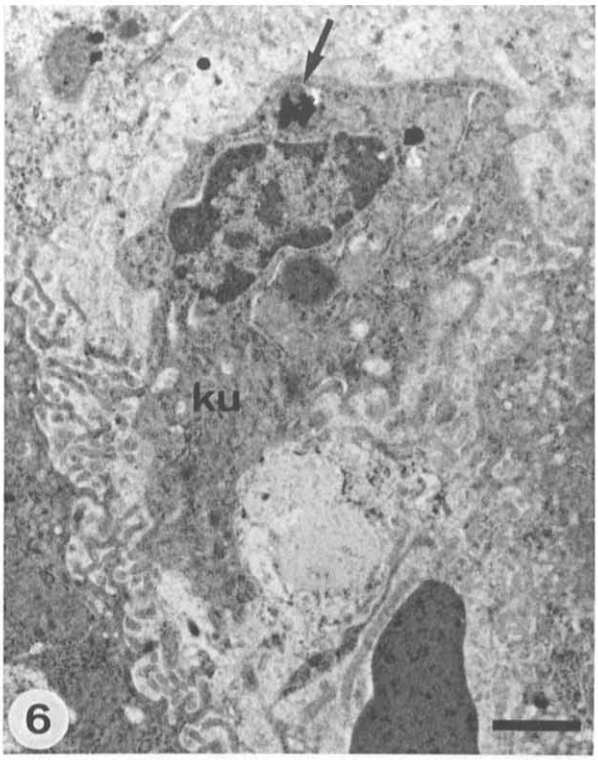
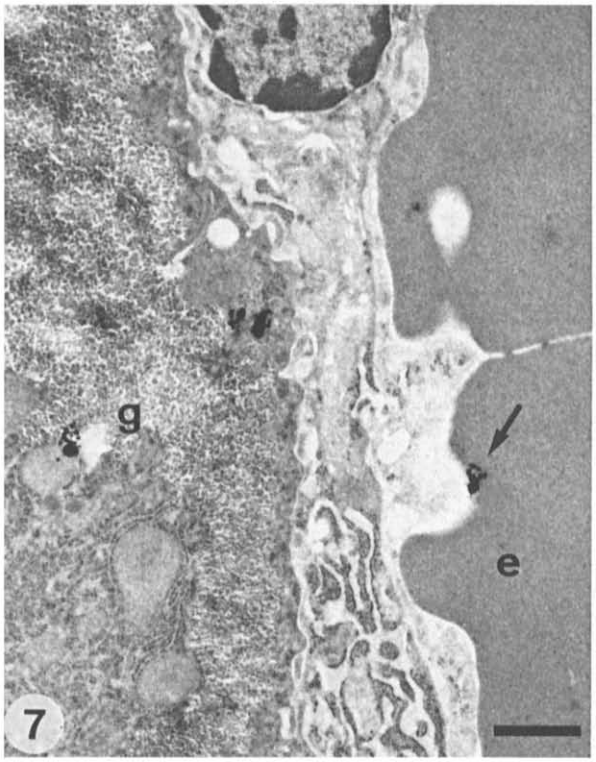


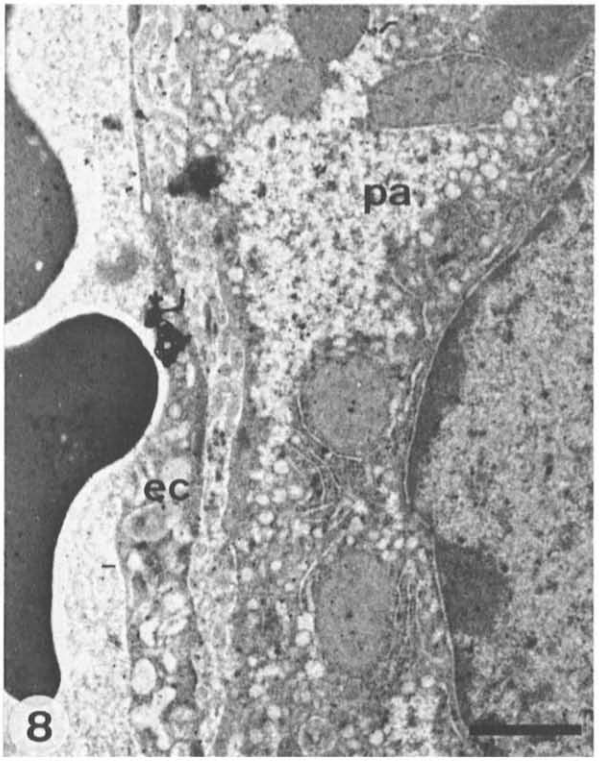
Fig. 3. System I: radioactivity from labeled diazepam is seen inside a nucleus (n) and in the glycogen storing region (arrow) of a parenchymal cell (pa). Furthermore, radioactivity is localized in the sinusoidal compartment. Bar = 1 μm.

Fig. 4. System I: typical cluster formation of radioactivity is seen inside a parenchymal cell. Discrete silver grains are also present in the glycogen storing region (g, arrow). Bar = 1 μm.


bc
5


ku
6


g
e
7


pa
ec
8

at all 3 time points inside parenchymal cells (Fig. 4) as well as in the sinusoidal lumen. This phenomenon is more accentuated for system I than for system II. Inside Kupffer cells such clustered RA is never found.

Five min after liposome administration, RA located in the vicinity of bile capillaries is rare, but after 6 h and 24 h, RA of [^{14}C]diazepam or its metabolites is found inside bile capillaries, indicating the way of elimination (Fig. 5). The percentage of total silver grains counted for each time interval in the parenchymal cells, including the space of Disse (arrow in Fig. 2) amounted to 81%, 93% and 92% (following the time course of 24 h) for the negative charged liposomes respectively to 82%, 92% and 86% for the sphingomyelin-containing liposomes. Maximal percentage of RA found in the sinusoidal compartment including Kupffer cells (Fig. 6), fat-storing cells and erythrocytes is found 5 min after administration and reached 19%. Minimal percentage of 7% is calculated after 6 h of administration for system I. After 24 h still 8% of silver grains are observed in the sinusoids in the case of system I and a rise of RA from 8% to 14% is documented for system II. At a closer view, RA situated in the sinusoids is often localized on the surfaces of the cells (Fig. 7). Sporadically silver spots are also found in endothelial cells lining up the sinusoidal compartment as in Fig. 8.

From analogous analysis of the [^{3}H]cholesteryl hexaydecyl ether liposome-invaded liver, similar results were obtained at all time points after i.v. administration. No significant differences in distribution and localization of the lipid-labeled systems (system I, respectively system II) could be found.

Discussion

Methodically, our data result from semiquantitative analysis of tissue pieces prepared for electron microscopic autoradiographs after each time interval. Excellent ultrastructural quality of autoradiographs and a high degree of preservation of lipids and the lipophilic drug is a prerequisite for precise localization of the components of i.v. administered liposomes. This is achieved using the glutaraldehyde – tannic acid – osmium tetroxide procedure which provides both fixing of lipids (especially phosphatidyl choline) and resistance to solvent extraction (Kalina and Pease, 1977; Saffitz et al., 1981; 1984). Tannic acid also increases the electron density of the surfaces of membranes of endothelial, Kupffer and fat-storing cells and of the sinusoidal lumen as well. In summary, this results in protecting adherence of any adsorbed material to these structures.

In the whole body autoradiograph (Fig. 1) a slight accumulation of [^{14}C]diazepam 5 minutes after administration is documented in the brain. Yet, RA is much lower when diazepam is incorporated into liposomes than after administration of free diazepam (Keller and Waser, 1984). No RA is found in the lungs in contrast to other small colloidal particles such as polyhexyl cyanoacrylate (Waser et al., 1987). As no redistribution of liposome-associated [^{14}C]diazepam from the liver after 6 and 24 h can be observed, the viability of these liposome carrier systems as slow release devices failed. The rise of RA from 8% up to 14% in the sinusoid after 24 h in electron microscopic autoradiographs may be explained by the elimination pathway of free rather than liposome-associated diazepam from the peripheral organs through the liver.

On the ultrastructural level we have shown that the lipophilic drug diazepam associated with small unilamellar liposomes is preferentially taken up by the liver parenchymal cells. No preference for either system I with negative charge nor system II containing sphingomyelin is observed. Interpretation of these results is tentative: since it is well known that the negatively charged liposomes are

← Fig. 5. System I: radioactivity is localized inside bile capillaries (bc, arrow). Bar = 1 μm.

Fig. 6. System II: to a lesser extent than inside parenchymal cells, radioactivity is also found, to be taken up by Kupffer cells (ku, arrow). Bar = 1 μm.

Fig. 7. System II: radioactivity can also be detected on the surface of erythrocytes (e); g, glycogen. Bar = 1 μm.

Fig. 8. System I: radioactivity is only rarely found in endothelial cells (ec) lining up the sinusoidal compartment. Bar = 1 μm.

cleared off from the blood stream much faster than neutrally and positively charged ones and on the other hand, that sphingomyelin has relatively high affinity to liver parenchymal cells (Alving et al., 1978; Scherphof et al., 1985), both systems may be equally qualified to penetrate the parenchymal cells. Taking into account that the yield of positive autoradiographs (showing silver grains) of the liver pieces excised for electron microscopic investigations after administration of system I is higher than that of system II, the data may be corrected in favour of system I. Nevertheless, we ascribe this fact to heterogeneity of the liver itself which may have physiological reasons with no qualitative relevance. In other words, uptake in different parts of the liver from the blood may be equal but distribution and incorporation into the functionally distinct cell types may be the same. Furthermore, in no case, RA can be detected in association with the membrane of parenchymal cells, neither in system I nor in system II. As other authors (Poste and Papahadjopoulos, 1976; Hoekstra et al., 1978) showed that uptake of negatively charged liposomes into isolated parenchymal cells occured by fusion, our lipid-associated drug diazepam would be expected to be incorporated into the membranes. Nevertheless, our results clearly show that after i.v. administration of [^{14}C]diazepam liposomes and [^{3}H] cholesteryl hexadecyl ether liposomes the label from both carrier systems is exclusively deposited inside the parenchymal cells.

Concerning the resolution power of the autoradiographic method on the electron microscopic level the attribution of silver spots to ultrastructural elements as cell membranes, mitochondria, nuclei and its membranes or lysosomes is unequivocally clear, although correlation of localization with biochemical and physiological aspects remains crucial.

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